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Regulatory T cell microRNA expression changes in children with acute Kawasaki disease

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Summary

Kawasaki disease (KD) is a type of systemic vasculitis syndrome related to immune dysfunction. Previous studies have implicated that dysfunctional regulatory T cells (T_{reg}) may be associated with the immune dysfunction in KD. In the absence of microRNAs (miRNAs), forkhead box protein 3 (FoxP3)$^+$ T_{reg} develop but fail to maintain immune homeostasis. This study was designed to investigate the effects of miR-155, miR-21 and miR-31 on T_{reg} in children with KD. The proportions of CD4$^+$CD25$^+$FoxP3$^+$ T_{reg} and the mean fluorescence intensity (MFI) of phosphorylated-signal transducer and activator of transcription (pSTAT)-5 and pSTAT-3 protein in CD4$^+$CD25$^+$ T_{reg} were analysed by flow cytometry. The concentration of interleukin (IL)-6 in plasma was measured by cytometric bead array. Real-time polymerase chain reaction was performed to detect the levels of microRNAs and associated factors in CD4$^+$CD25$^+$ T_{reg}. The proportion of T_{reg} and the mRNA levels of the associated factors [FoxP3, glucocorticoid-induced tumour necrosis factor-receptor (GITR), cytotoxic T lymphocyte antigen (CTLA)-4)] were significantly lower in KD patients ($P < 0{\cdot}05$). MiR-155 and miR-21 levels were significantly down-regulated and miR-31 expression was higher in KD patients ($P < 0{\cdot}05$). Plasma interleukin (IL)-6 concentrations, pSTAT-3 protein levels and suppressors of cytokine signalling (SOCS)-1 mRNA expression were remarkably elevated in acute KD ($P < 0{\cdot}05$), while pSTAT-5 protein levels were remarkably decreased in acute KD ($P < 0{\cdot}05$). These findings were reversed after intravenous immunoglobulin treatment ($P < 0{\cdot}05$). Our results demonstrate that FoxP3 mRNA levels were primarily affected by the miR-155/SOCS1 and the miR-31 signalling pathways. These results suggest that the decrease in FoxP3$^+$ T_{reg} might be associated with decreased expression of miR-155, leading to aberrant SOCS1/STAT-5 signalling and overexpression of miR-31 in patients with acute KD.

Keywords: Kawasaki disease, miR-155, miR-21, miR-31, T_{reg} cells

Introduction

Kawasaki disease (KD) is an acute systemic vasculitis syndrome, most frequently affecting infants and children. Previous studies have shown that an aberrant immune response is involved in the pathogenesis of KD, but the mechanisms resulting in immune dysfunction remain unknown [1–3]. CD4$^+$CD25$^+$forkhead box protein 3 (FoxP3$^+$) regulatory T cells (T_{reg}) are critical to maintain autoimmune tolerance and immune homeostasis. The transcription factor FoxP3 is required for T_{reg} development and function and is considered the 'master regulator' of T_{reg} [4–6]. Several studies have reported that the numbers of T_{reg} decrease and their functional properties are compromised during the acute phase of KD [7,8]. However, the decrease in T_{reg} numbers has also been shown to partially recover in response to intravenous immunoglobulin (IVIG) treatment.

It has been suggested that dysfunctional T_{reg} are associated with immune dysfunction in KD, but the mechanisms resulting in T_{reg} dysfunction remain unknown [7,8]. A class of small non-coding RNA known as microRNAs (miRNA) has been implicated in the regulation of the expression of

Table 1. Characteristics of patients with Kawasaki disease.

	KD
Number of subjects	33
Male/female, *n* (%)	24/09 (72·7)
Age in months, median (range)	12 (3–54)
Duration of fever (days), median (range)	7 (5–10)
Rash, *n* (%)	30 (90·9)
Lymphadenopathy, *n* (%)	31 (93·9)
Conjunctival congestion, *n* (%)	29 (87·9)
Oral mucosal changes, *n* (%)	30 (90·9)
Peeling, *n* (%)	30 (90·9)
Arthritis, *n* (%)	1 (3·0)
Coronary artery lesion, *n* (%)	10 (30·3)
Aneurysm, *n* (%)	1 (3·0)
Thrombocytosis, *n* (%)	30 (90·9)
Pyuria, *n* (%)	1 (3·0)
Jaundice, *n*	0
Giant peripheral aneurysm, *n*	0

Duration of fever (days), median (range): mean duration of fever from the onset of disease to the day of sample collection prior to intravenous immunoglobulin (IVIG) treatment. Giant aneurysm (ANl): luminal diameter > 8 mm, in older children (≥5 years), lesions with luminal diameter > four times that of the normal coronary artery.

genes essential for cellular differentiation, homeostasis and function through target mRNA degradation or translational control [9,10]. Expression of certain miRNAs in FoxP3$^+$ T_{reg} is required to maintain the body's immune tolerance to self, thereby preventing autoimmunity [9]. However, it is unknown whether changes in miRNA expression alter FoxP3 expression in acute KD.

FoxP3 plays a central role in establishment of the T_{reg} cell lineage, and the potential link between FoxP3 and miR-155 expression suggests that miR-155 may also function in regulating T_{reg} cell differentiation, maintenance or function [11,12]. Moreover, FoxP3-dependent miR155 regulation maintains the competitive fitness of a subset of T_{reg} by targeting suppressors of cytokine signalling (SOCS)-1 [11]. miR-31 and miR-21 both affect FoxP3 expression levels. In addition, signal transducer and activator of transcription (STAT)-3 suppresses miR-21 expression, which induces the down-regulation of FoxP3 expression [13–16].

Thus, miR-155, miR-21 and miR-31 signal pathways affect FoxP3 expression levels. In this study, we examined the expression levels of miR-155, miR-21, miR-31 and their associated signalling molecules to investigate further the possible mechanisms of T_{reg} down-regulation in KD.

Materials and methods

Subjects

Thirty-three children in the acute febrile stage of KD (24 males and nine females; median age: 12 months; age range: 3–54 months) were enrolled into this study (Table 1). The study participants were recruited from the in-patient paediatric population at Shenzhen Children's Hospital between November 2012 and May 2013. KD diagnosis was made according to the clinical criteria published by the Kawasaki Disease Research Committee of Japan. Fever onset was considered as the first day of an acute KD phase. Samples were obtained at the acute stage (range: 5–10 days; median: 7 days) before IVIG treatment. Post-treatment samples were also obtained after IVIG therapy (range: 7–13 days; median: 8 days). Fourteen similarly age-matched children (eight males and six females; median age: 16 months; age range: 4–38 months) who were physically healthy, without any clinical signs of infection or inflammation, consented to participate as healthy controls (Ctrl).

Informed consent was obtained from the parents of all patients as a condition of study enrolment, and the study was carried out with pre-approval by the local Medical Ethics Committee. Blood samples were analysed immediately without stimulation of mitogens or culture *in vitro*, except where indicated. All patients with KD received 2 g/kg IVIG and were administered aspirin orally. Thirty-two of the KD patients responded favourably to the IVIG infusion, but one patient's fever persisted for 72 h after IVIG treatment; therefore, the patient was given a second dose of IVIG (Table 2). All the KD patients received their first two-dimensional echocardiographic examination within 10 days. Coronary artery lesion (CAL) was defined by an arterial internal diameter > 3 mm (<5 years) or > 4·0 mm (≥5 years) or by the presence of coronary artery aneurysms. Coronary artery aneurysms were considered 'present' if the maximum internal lumen diameter was enlarged by at least 1·5-fold. Patients with KD were divided into the KD-CAL$^+$ group and KD-CAL$^-$ group according to the echocardiographic examination results.

Blood samples

Venous blood (5 ml) was collected from each of the KD patients and each of the healthy controls and stored in ethylenediamine tetraacetic acid (EDTA)-Na_2 as an anti-

Table 2. Clinical data of patients with acute Kawasaki disease (KD) before and after intravenous immunoglobulin (IVIG) treatment.

Groups	*n*	WBC ($\times 10^9$ /l)	*n*% (%)	PLT ($\times 10^9$ /l)	CRP (mg/l)	PCT (ng/ml)	sIL-2R (pg/ml)
KD	33	16·1 ± 6·6	61·2 ± 14·1	398 ± 159	65·1 ± 37·7	0·57 ± 0·53	24 459·6 ± 12 978·5
KD^{IVIG}	33	7·4 ± 1·6*	49·8 ± 9·1*	306 ± 75*	10·5 ± 5·9*	0·25 ± 0·20*	16 015·7 ± 8367·6*

*$P < 0·05$. Values are expressed as mean ± standard deviation. KD *versus* KD^{IVIG}. CRP: C-reactive protein; N: neutrophils; PCT: procalcitonin; PLT: platelet; sIL-2R: soluble interleukin-2 receptor; WBC: white blood cell.

Table 3. Primers for real-time polymerase chain reaction (PCR).

Gene	Primer sequence	Annealing temperature, °C	Product size, bp
FoxP3	Sense: 5′-GGAAAGGAGGATGGACGAAC-3′ Anti-sense: 5′-GCAGGCAAGACAGTGGAAAC-3′	56	122
GITR	Sense: 5′-ACACGCACTTCACCTGGGTCG-3′ Anti-sense: 5′-TGTGCCATGCTCGGGTTTCA-3′	56	129
CTLA-4	Sense: 5′-GTCCGGGTGACAGTGCTTCG-3′ Anti-sense: 5′-CCAGGTAGTATGGCGGTGGG-3′	56	220
SOCS-1	Sense: 5′-CCGATTACCGGCGCATCACG -3′ Anti-sense: 5′-TGGGTCCCGAGGCCATCTTCAC-3′	56	186
GAPDH	Sense: 5′-CAAGAAGGTGGTGAAGCAGG-3′ Anti-sense: 5′-AGGTGGAGGAGTGGGTGTCG-3′	54–60	110

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; SOCS: suppressors of cytokine signalling; CTLA: cytotoxic T lymphocyte antigen; GITR: glucocorticoid-induced tumour necrosis factor-receptor; FoxP3: forkhead box protein 3; bp: base pairs.

coagulant. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient for subsequent flow cytometric analysis. Plasma was obtained after centrifugation and stored at −80°C for analysis by cytometric bead array (CBA). $CD4^+CD25^+$ T cells were isolated immediately from the patients' peripheral blood samples, according to the manufacturer's instructions for microbeads (11363D, Dynal; Invitrogen, Carlsbad, CA, USA). Cell populations were considered pure at > 97% by flow cytometry, and cell activity was investigated using the trypan blue exclusion assay (using the threshold of > 95% of the cell population as a positive indicator of significantly decreased cell activity).

Total RNA extraction and cDNA synthesis

Total RNA (including miRNAs) was isolated from $CD4^+CD25^+$ T cells according to the manufacturer's instructions using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). The integrity of the isolated total RNA was confirmed by an average optical density $(OD)_{260}/OD_{280}$ nm absorption ratio of 1·98. cDNA was synthesized with oligo-dT primers and RevertAid™ H Minus reverse transcriptase (Fermentas, Vilnius, Lithuania). The miRNA cDNA was synthesized with the miScript II RT Kit from Qiagen. Negative control samples (no first-strand synthesis) were prepared by performing reverse transcription in the absence of reverse transcriptase.

LightCycler real-time polymerase chain reaction (PCR)

The cDNA levels of FoxP3, glucocorticoid-induced tumour necrosis factor receptor-related protein (GITR), cytotoxic T lymphocyte antigen 4 (CTLA-4) and SOCS-1 were quantitated by real-time PCR using the Quantitect™ SYBR green PCR Kit (Takara, Kyoto, Japan) and a LightCycler® 2·0 (Roche Molecular Biochemicals, Basel, Switzerland). The primers used for the real-time PCR assays are listed in Table 3. The cDNA levels of miR-155, miR-21 and miR-31 were quantitated by real-time PCR using the miScript SYBR Green PCR Kit (Qiagen) and the LightCycler® 2·0. The primers used for miRNA real-time PCR were designed according to the recommendations for use with the miRNA miScript Primer Assay (Qiagen). The second derivative maximum method was performed for CP determination using LightCycler software version 3·5·30 (Roche Molecular Biochemicals). After normalization with Relative Quantification Software version 1·0 (Roche Molecular Biochemicals), the final results were calculated as ratios of the target genes or miRNAs to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6.

Flow cytometry analysis of T_{reg}

Whole blood samples were processed for analysis by first incubating with anti-human CD4-eFluor 450 and anti-human CD25-peridinin chlorophyll-cyanin 5·5 (PerCP-Cy5·5) (eBioscience, San Diego, CA, USA) at 4°C for 30 min. The cells were then fixed and permeabilized (according to the manufacturer's instructions) and stained with anti-human FoxP3-allophycocyanin (APC) (eBioscience), anti-mouse STAT-5-Alexa Fluor 488 and anti-mouse STAT-3-phycoerythrin (PE) (BD Pharmingen, San Diego, CA, USA). Isotype controls were used for normalization purposes and to confirm antibody specificity. T_{reg} proportions and mean fluorescence intensity (MFI) for phosphorylated (p)STAT-5 and pSTAT-3 protein in $CD4^+CD25^+$ T cells were analysed using a FACSCantoII cytometer equipped with FACS Diva version 6·1·3 software (Beckman Coulter, Fullerton, CA, USA).

CBA detection of plasma IL-6

The plasma levels of interleukin (IL)-6 were measured using a CBA kit (eBioscience), according to the manufacturer's instructions. All samples were measured in duplicate.

Statistical analysis

SPSS version 13·0 software for Windows was used for statistical analysis (SPSS Inc., Chicago, IL, USA). The data are expressed as mean ± standard deviation (s.d.). Intergroup

differences were assessed by Student's *t*-test. Factor analysis and multiple linear regression analysis were applied to detect correlations between different study parameters. *P*-values < 0·05 were considered indicative of statistical significance.

Results

Acute KD patients have decreased numbers of $CD4^+$ $CD25^+FoxP3^+$ T_{reg} and decreased expression of associated factors

The number of T_{reg} from patients' whole blood was quantified by flow cytometry (Fig. 1). The proportion of peripheral T_{reg} in KD patients' whole blood was significantly lower than that of healthy subjects ($1{\cdot}25 \pm 0{\cdot}78$ *versus* $3{\cdot}96 \pm 0{\cdot}29\%$, $t = 17{\cdot}14$, $P < 0{\cdot}05$), and increased gradually after IVIG treatment ($3{\cdot}59 \pm 0{\cdot}96\%$, $t = 10{\cdot}79$, $P < 0{\cdot}05$) (Fig. 1a,b). We performed real-time PCR to evaluate the expression levels of FoxP3, GITR and CTLA-4 in $CD4^+CD25^+$ T cells from patients with KD. As shown in Fig. 1c, the expression levels of FoxP3, GITR and CTLA-4 mRNA were remarkably lower in the KD group compared to the Ctrl group (FoxP3: $(1{\cdot}32 \pm 0{\cdot}62$ *versus* $3{\cdot}39 \pm 0{\cdot}97) \times 10^{-1}$, $t = 8{\cdot}83$, $P < 0{\cdot}05$; GITR: $(3{\cdot}36 \pm 1{\cdot}04$ *versus* $42{\cdot}4 \pm 19{\cdot}6) \times 10^{-4}$, $t = 7{\cdot}47$, $P < 0{\cdot}05$; CTLA-4: $(4{\cdot}88 \pm 1{\cdot}25$ *versus* $12{\cdot}9 \pm 2{\cdot}36) \times 10^{-2}$, $t = 12{\cdot}00$, $P < 0{\cdot}05$). However, the expression level of each gene increased significantly after IVIG treatment compared to pretreatment levels [FoxP3: $(3{\cdot}13 \pm 0{\cdot}93) \times 10^{-1}$, $t = 9{\cdot}24$, $P < 0{\cdot}05$; GITR: $(43{\cdot}7 \pm 8{\cdot}37) \times 10^{-4}$, $t = 27{\cdot}45$, $P < 0{\cdot}05$; CTLA-4: $(10{\cdot}1 \pm 2{\cdot}89) \times 10^{-2}$, $t = 9{\cdot}47$, $P < 0{\cdot}05$]. We did not detect any significant difference between the $KD\text{-}CAL^+$ and $KD\text{-}CAL^-$ groups, but the trend was that the $KD\text{-}CAL^+$ group had decreased proportions of T_{reg} and decreased expression of associated factors compared to the $KD\text{-}CAL^-$ group [T_{reg}: $(1{\cdot}26 \pm 0{\cdot}75\%$ *versus* $1{\cdot}28 \pm 0{\cdot}81\%$, $t = 0{\cdot}07$, $P > 0{\cdot}05$); FoxP3: $(1{\cdot}14 \pm 0{\cdot}54$ *versus* $1{\cdot}39 \pm 0{\cdot}64) \times 10^{-1}$, $t = 1{\cdot}08$, $P > 0{\cdot}05$; GITR: $(2{\cdot}96 \pm 1{\cdot}13$ *versus* $3{\cdot}53 \pm 0{\cdot}98) \times 10^{-4}$, $t = 0{\cdot}85$, $P > 0{\cdot}05$; CTLA-4: $(3{\cdot}89 \pm 1{\cdot}68$ *versus* $4{\cdot}62 \pm 0{\cdot}97) \times 10^{-2}$, $t = 1{\cdot}57$, $P > 0{\cdot}05$].

The expression levels of miR-155, miR-21 and miR-31 in KD patients

It has been shown previously that miR-155, miR-21 and miR-31 have important effects on FoxP3 expression. Because we saw decreased expression of FoxP3 in acute KD, we wanted to determine whether or not the associated miRNAs were also decreased. As shown in Fig. 2, the levels of miR-155 and miR-21 were down-regulated significantly compared to controls during the acute phase of KD [miR-155: $(9{\cdot}83 \pm 2{\cdot}82$ *versus* $22{\cdot}90 \pm 8{\cdot}15) \times 10^{-3}$, $t = 5{\cdot}84$, $P < 0{\cdot}05$; miR-21: $(5{\cdot}86 \pm 2{\cdot}37$ *versus* $17{\cdot}67 \pm 5{\cdot}04)$, $t = 14{\cdot}23$, $P < 0{\cdot}05$]. IVIG treatment significantly rescued KD miRNA levels, but not up to control levels [miR-155: $(13{\cdot}00 \pm 5{\cdot}46) \times 10^{-3}$ $t = 3{\cdot}00$, $P < 0{\cdot}05$; miR-21: $(8{\cdot}95 \pm 4{\cdot}82)$, $t = 3{\cdot}30$, $P < 0{\cdot}05$]. miR-31 expression was significantly up-regulated compared to controls [$(32{\cdot}60 \pm 13{\cdot}05$ *versus* $12{\cdot}90 \pm 6{\cdot}87) \times 10^{-4}$, $t = 6{\cdot}77$, $P < 0{\cdot}05$]. IVIG treatment partially rescued miR-31 expression, but not to control levels [$(24{\cdot}10 \pm 11{\cdot}70) \times 10^{-4}$, $t = 3{\cdot}04$, $P < 0{\cdot}05$].

The up-regulated genes in patients with acute KD are associated with miR-155 signalling

MiR-155 positively regulates FoxP3 mRNA expression by inhibiting the SOCS-1/STAT-5 signalling pathway [11,12] (Fig. 4). Thus, we examined the levels of miR-155-related signalling molecules by flow cytometry and subsequent real-time PCR (Fig. 3). SOCS-1 mRNA expression was elevated significantly in KD samples, while pSTAT-5 protein levels were decreased significantly in the KD group compared to the Ctrl group [SOCS-1: $(7{\cdot}40 \pm 3{\cdot}42$ *versus* $3{\cdot}29 \pm 1{\cdot}08) \times 10^{-2}$, $t = 6{\cdot}21$, $P < 0{\cdot}05$; pSTAT-5: $(33{\cdot}48 \pm 15{\cdot}39$ *versus* $56{\cdot}29 \pm 11{\cdot}66)$, $t = 4{\cdot}96$, $P < 0{\cdot}05$]. IVIG treatment rescued SOCS-1 mRNA and pSTAT-5 protein levels to control levels [SOCS-1: $(4{\cdot}12 \pm 1{\cdot}81) \times 10^{-2}$, $t = 4{\cdot}90$, $P < 0{\cdot}05$; pSTAT-5: $(52{\cdot}73 \pm 18{\cdot}43)$, $t = 4{\cdot}60$, $P < 0{\cdot}05$] (Fig. 3a–d).

miR-21-related signalling molecules are up-regulated in acute KD patients

The IL-6/STAT-3 signalling pathway is involved in the down-regulation of miR-21, and miR-21 positively regulates FoxP3 mRNA levels [13–16] (Fig. 4). We sought to determine if KD patients had altered IL-6/STAT-3 signalling as a result of changes in miR-21 levels. We detected plasma levels of IL-6 and pSTAT-3 by CBA and flow cytometry (Fig. 3a,c,e) and found that plasma IL-6 concentrations and pSTAT-3 protein levels were elevated significantly during acute KD (IL-6: $52{\cdot}80 \pm 35{\cdot}55$ *versus* $5{\cdot}92 \pm 1{\cdot}11$, $t = 7{\cdot}57$, $P < 0{\cdot}05$; pSTAT-3: $24{\cdot}72 \pm 9{\cdot}22$ *versus* $9{\cdot}14 \pm 2{\cdot}98$, $t = 8{\cdot}70$, $P < 0{\cdot}05$). IVIG treatment resulted in a dramatic decrease in IL-6 and pSTAT-3 levels (IL-6: $6{\cdot}14 \pm 2{\cdot}48$, $t = 7{\cdot}52$, $P < 0{\cdot}05$ compared to pretreatment levels; pSTAT-3: $12{\cdot}67 \pm 5{\cdot}07$, $t = 6{\cdot}59$, $P < 0{\cdot}05$ compared to pretreatment levels) (Fig. 3c,e).

According to multiple linear regression analysis, miR155 and miR-31 control FoxP3 mRNA levels

This study explored the relationship between the miRNA signalling pathways and FoxP3 mRNA. Although each of the elements in the pathways may be co-linear, the outputs from the different pathways should vary independently. Factor analysis is a statistical method to explore how to

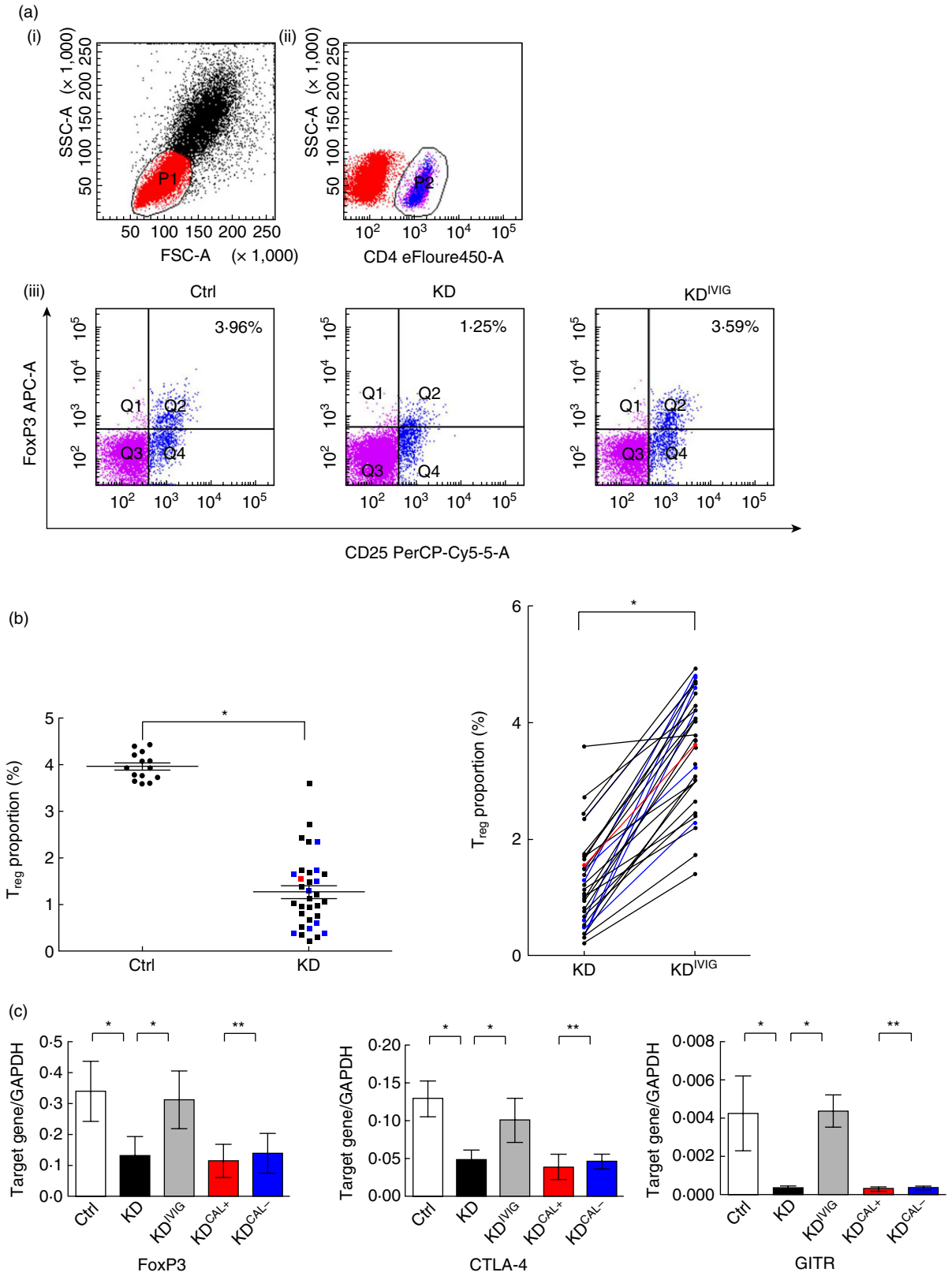

Fig. 1. The expression of $CD4^+$ $CD25^+$forkhead box P3 (FoxP3)$^+$ regulatory T cells (T_{reg}) and T_{reg}-related factors from patients with Kawasaki disease (KD). Thirty-three KD patients and 14 control subjects were enrolled into this study. All KD patients received intravenous immunoglobulin (IVIG) treatment. Ten patients presented with coronary artery lesions (KD-CAL^+), as measured by echocardiography. (a) Flow cytometry histogram for $CD4^+CD25^+FoxP3^+$ T_{reg}. (i) Peripheral lymphocytes were gated in the dot-plot of side-scatter *versus* P1. (ii) $CD4^+$ lymphocytes were gated in the dot-plot of side-scatter *versus* P2. (iii) Analysis of peripheral $CD4^+CD25^+FoxP3^+$ T_{reg} from healthy controls, patients with KD and KD patients after IVIG treatment (KD^{IVIG}) were gated in $CD4^+$ lymphocytes. (b) The proportions of $CD4^+$ $CD25^+FoxP3^+$ T_{reg}. (c) The expressions of T_{reg}-related factors. Relative expressions were determined by real-time polymerase chain reaction (PCR) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous reference gene. Normalized values are derived from the ratios of target gene/GAPDH mRNA expression. Data are shown as mean ± standard deviation. Statistical differences between the two populations were determined using a Student's *t*-test. $^*P < 0{\cdot}05$; $^{**}P > 0{\cdot}05$. Ctrl: healthy control; KD^{IVIG}: IVIG treatment. Red dot: subjects with coronary artery aneurysm; blue dot: subjects with dilated coronary arteries; black dot: subjects with normal coronary arteries.

describe the research object by a number of factors. We cannot measure directly whether interesting common factors are independent of each other, because each common factor has its own significance that does not overlap with other factors. To understand more clearly the relationship between FoxP3 mRNA levels and miR-155, miR-31 and miR-21-associated signalling molecules in acute KD, data from the KD group were selected for factor analysis and multiple linear regression analysis. As shown in Table 4, the proportion of variance and eigenvalues of factor 1 were 31·819 and 2·227%, respectively, the proportion of variance and eigenvalues of factor 2 were 27·297 and 1·911%, respectively, and the proportion of variance and eigenvalues of factor 3 were 21·251 and 1·488%, respectively. Factor 1 mainly controls IL-6, pSTAT-3 and miR-21 expression, factor 2 mainly controls miR-155 and SOCS-1 expression and factor 3 mainly controls miR-31 expression. Based on the factor score coefficients calculated for each sample predictive value affecting FoxP3 mRNA expression, we generated the following predictive model:

- factor 1 = 0·909 × pSTAT-3–0·832 × miR-21 + 0·821 × IL-6.
- factor 2 = 0·902 × SOCS-1–0·839 × miR-155.
- factor 3 = 0·927 × miR-31.

As shown in Table 5, multiple linear regression analysis revealed that the miR-155/SOCS-1 and miR-31 signalling pathways ($R^2_C = 0{\cdot}652$) influence FoxP3 mRNA levels. According to the known standardized partial regression coefficient, the influence sizes were miR-31 (Beta = –37·219) > miR-155/SOCS-1 (Beta = –0·628).

Discussion

Kawasaki disease is a type of systemic vasculitis syndrome related to immune dysfunction. The immunopathogenesis of KD remains unknown, and requires further study [1–3]. T_{reg} constitute a developmentally and functionally distinct T cell subpopulation required for sustained immunological self-tolerance and homeostasis. The transcription factor FoxP3 is expressed specifically in T_{reg} and is a key regulator of their differentiation and suppressor function [4–6]. In several autoimmune diseases, the number of T_{reg} cells decreases, and adoptive transfer of purified T_{reg} cells has been shown to improve autoimmune disorders [17,18]. Recent data in humans suggest that T_{reg} cells play an important role in the pathogenesis of a diverse group of immune-mediated diseases, including psoriasis, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and asthma [19–21]. Several studies have reported that T_{reg} number and

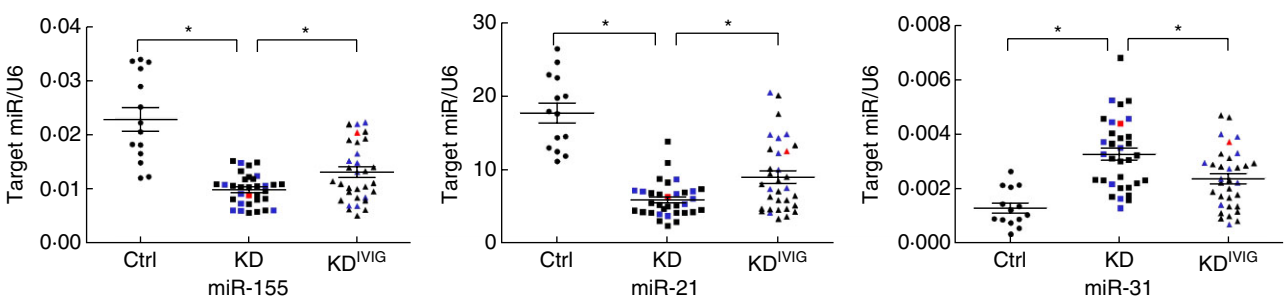

Fig. 2. The expression of microRNAs (miRNAs) in $CD4^+$ $CD25^+$ T cells from Kawasaki disease (KD) patients. Relative expression levels of miRNAs were determined by real-time polymerase chain reaction (PCR) using U6 as an endogenous reference. Normalized values are derived from the ratios of target miR/U6 expression, shown as mean ± standard deviation. Statistical differences between the control and KD patients were determined using a Student's *t*-test. $^*P < 0{\cdot}05$. Ctrl: healthy control; KD^{IVIG}: intravenous immunoglobulin (IVIG) treatment. Red dot: subjects with coronary artery aneurysm; blue dot: subjects with dilated coronary arteries; black dot: subjects with normal coronary arteries.

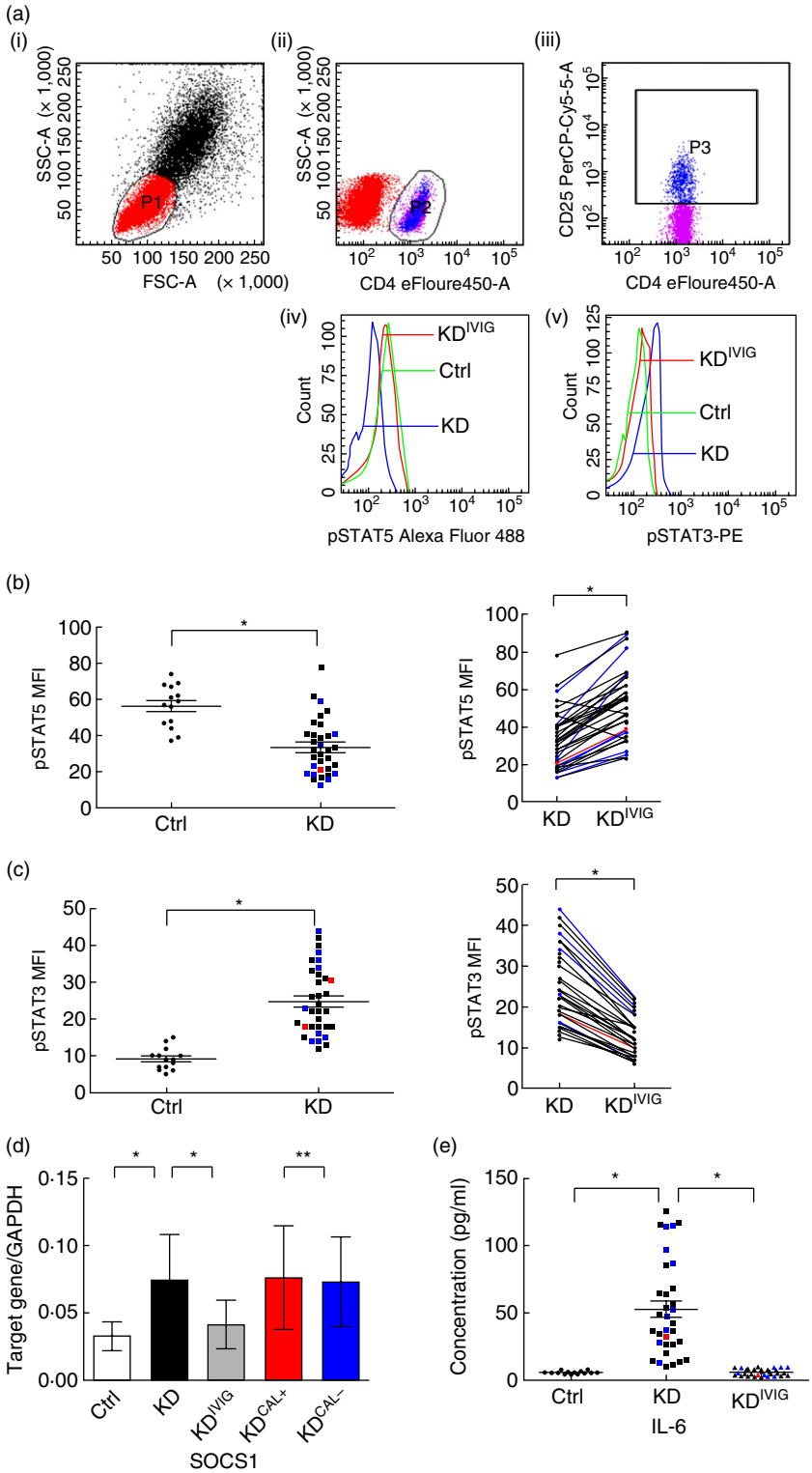

Fig. 3. The expression of microRNA (miRNA)-related signalling molecules in Kawasaki disease (KD) patients. (a) Flow cytometry histogram for phosphorylated signal transducer and activator of transcription (pSTAT)-5 and pSTAT-3. (i) Peripheral lymphocytes were gated in the dot-plot of side-scatter *versus* P1. (ii) $CD4^+$ lymphocytes were gated in the dot-plot of side-scatter *versus* P2. (iii) $CD4^+$ $CD25^+$ lymphocytes were gated in the dot-plot of side-scatter *versus* P3. (iv) The mean fluorescence intensity (MFI) for pSTAT-5 and pSTAT-3 protein in healthy controls, patients with KD and intravenous immunoglobulin (IVIG) treatment (KD^{IVIG}) were gated in $CD4^+$ $CD25^+$ lymphocytes. (v) The MFI for pSTAT-3 protein in healthy control, patients with KD and KD^{IVIG} were gated in $CD4^+$ $CD25^+$ lymphocytes. (b) The MFI for pSTAT-5 in $CD4^+CD25^+$ T cells. (c) The MFI for pSTAT-3 protein in $CD4^+CD25^+$ T cells. (d) SOCS-1 mRNA expression of in KD patients compared to Ctrl. Relative expression was determined by real-time polymerase chain reaction (PCR) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous reference gene. Normalized values are derived from the ratios of target gene/GAPDH mRNA expression. (e) Plasma concentrations of interleukin (IL)-6. Data are shown as mean ± standard deviation. Statistical differences were determined using a Student's *t*-test. $^*P < 0{\cdot}05$; $^{**}P > 0{\cdot}05$. Ctrl: healthy control; KD^{IVIG}: intravenous immunoglobulin (IVIG) treatment. Red dot: subjects with coronary artery aneurysm; blue dot: subjects with dilated coronary arteries; black dot: subjects with normal coronary arteries.

function is low in acute KD, but recover somewhat after IVIG treatment [7,8]. Consistent with previous findings, our data demonstrate that T_{reg} numbers and the expression of T_{reg}-related factors (FoxP3, GITR, CTLA-4) were decreased significantly in KD patients. IVIG treatment resulted in partial recovery of T_{reg} number and gene expression, but the mechanism remains unclear. Notably, one patient in our study had a higher proportion of T_{reg} compared to the other KD patients but was unresponsive to IVIG treatment, even after a second treatment. This patient

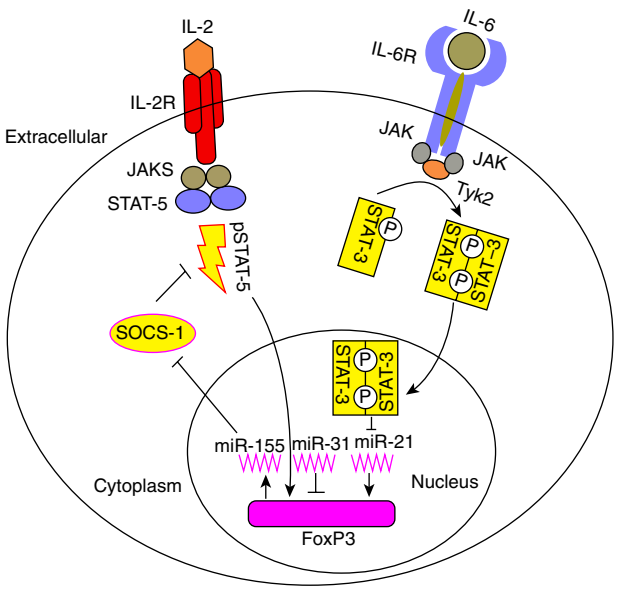

Fig. 4. MiR-155, miR-21 and miR-31 are involved in maintaining $CD4^+$ $CD25^+$forkhead box P3 (FoxP3)$^+T_{reg}$. FoxP3 directly promotes miR-155 transcription, and the increased miR-155 binds to the 3′ UTR of suppressors of cytokine signalling (SOCS)-1 mRNA to suppress its protein expression. Thus, mIR-155 indirectly activates signal transducer and activator of transcription (STAT)-5 to maintain FoxP3 expression. MiR-31 negatively regulates FoxP3 expression by directly binding to its potential target site in the FoxP3 mRNA 3′ UTR. Interleukin (IL)-6 may induce STAT-3 activation, while STAT-3 inhibits miR-21 expression. MiR-21 positively regulates FoxP3 expression.

was in fever for more than 72 h after the first IVIG treatment. We hypothesize that altered T_{reg} function may account for this patient's IVIG resistance; however, further study is required to verify this.

miRNAs are small conserved non-coding RNA molecules that regulate gene expression post-transcriptionally by targeting the 3′ untranslated region (UTR) of specific mRNAs for degradation or translational repression [9,10]. There is an increasing number of reports that miRNAs are involved in the pathogenesis of various human autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis [22,23]. Several studies have reported that miRNAs are important for T_{reg} immune function, as miRNA depletion specifically in the $FoxP3^+$ T_{reg} cell lineage caused fatal autoimmune disease. miRNAs play a key role in T_{reg} homeostasis and function [9,24–26], and may participate in KD pathogenesis [27]. However, previous studies had not examined whether aberrant expression of miRNAs in KD patients results in decreased pools of $FoxP3^+$ T_{reg} cells. In this study, we observed that the levels of miR-155 and miR-21 were decreased significantly and the level of miR-31 expression was increased significantly during acute KD. The above-mentioned changes in miR expression were reversed significantly by IVIG treatment. To understand more clearly the relationship between aberrant miRNA expression and changes in expression of FoxP3 and other factors in KD patients, we performed factor analysis. We found that factors controlling miR-21, miR-155 and miR-31 expression also influence FoxP3 mRNA expression. Therefore, we propose that the decreased population of $FoxP3^+$ T_{reg} in KD patients may be correlated with the decreased expression of miR-155 and miR-21 and the overexpression of miR-31.

We further investigated the mechanisms resulting in abnormal miRNA expression in children with acute KD. Previous studies have shown that miR-155 is highly expressed in T_{reg}; miR-155 is regulated directly by FoxP3 and may be involved in regulating T_{reg} differentiation, main-

Table 5. Multiple linear regression analysis of forkhead box protein 3 (FoxP3) microRNA and factor 1, factor 2 and factor 3.

Group	B (regression coefficient)	Beta (standardized regression coefficient)	t	*P*
Factor 1	1·106E-05	0·007	0·062	0·951
Factor 2	−0·628	−0·330	−3·153	0·004
Factor 3	−37·219	−0·728	−6·826	0·000

We first calculated the factor score coefficients for each sample to predict each factor's effect on FoxP3 mRNA expression. A residual analysis test was used to test whether each factor meets the conditions for multivariate linear regression analysis. Finally, we performed stepwise regression analysis to assess the relationships between FoxP3 mRNA expression and factor 1, factor 2 and factor 3.

Table 4. Factor analysis of components affecting forkhead box protein 3 expression in acute Kawasaki disease.

			Component (extraction factor)			
Factor	% of variance	Cumulative %	Factor 1 loadings	Factor 2 loadings	Factor 3 loadings	Communalities
pSTAT-3	31·819	31·819	0·909			0·864
miR-21			−0·832			0·752
IL-6			0·821			0·693
SOCS-1	27·297	59·116		0·902		0·816
miR-155				−0·839		0·817
pSTAT-5						0·800
miR-31	21·251	80·367			0·927	0·884
Eigenvalues			2·227	1·911	1·488	

SOCS: suppressors of cytokine signalling; IL: interleukin; pSTAT: phosphorylated signal transducer and activator of transcription.

tenance or function [11,12]. Over-expression of miR-155 in T_{reg} resulted in decreased SOCS-1 expression and enhanced activation of STAT-5 [11,12]. Binding of phosphorylated STAT-5 to the FoxP3 promoter increases FoxP3 transcription, resulting in increased FoxP3 protein in T_{reg} [28]. Here, we found that SOCS-1 mRNA levels were increased significantly in acute KD, while pSTAT-5 protein levels decreased significantly. IVIG treatment substantially rescued SOCS-1 mRNA and pSTAT-5 protein levels. Multiple linear regression analysis showed that factor 2 mainly controls miR-155/SOCS-1 expression, thus affecting FoxP3 mRNA expression. Therefore, we speculate that aberrant SOCS-1/STAT-5 signalling resulting from decreased miR-155 might be one mechanism of the down-regulation of $FoxP3^+$ T_{reg} in acute KD.

Redouane *et al*. reported recently that miR-31 and miR-21 both regulate FoxP3. MiR-31 regulates FoxP3 expression negatively by binding directly to its potential target site in the 3′ UTR of FoxP3 mRNA [13]. In this study, miR-31 expression affected FoxP3 mRNA levels negatively. Multiple linear regression analysis showed that factor 3 mainly controls miR-31, which affects FoxP3 mRNA expression. According to the known standardized partial regression coefficient, miR-31 had a larger effect on FoxP3 mRNA levels than the miR-155/SOCS-1 signalling pathway. MiR-21 regulates FoxP3 expression positively, but no potential target sequence in FoxP3 mRNA has been identified. Redouane *et al*. suggested that the mechanism of FoxP3 up-regulation may be indirect [13]. The inflammatory cytokine IL-6 suppresses T_{reg} development and function [14]. IL-6 induces STAT-3 activation, while STAT-3 suppresses the expression of miR-21 [14–16]. Our data showed that IL-6 and pSTAT-3 levels in KD were significantly higher compared to healthy controls. IVIG treatment decreased the levels of IL-6 and pSTAT-3 significantly. Thus, it is feasible that aberrant IL-6/STAT-3 signalling decreases miR-21 levels, which subsequently reduces the number of $FoxP3^+$ T_{reg} in acute KD. Our experimental data support this hypothesis, but multivariate regression analysis revealed that factor 1, which mainly controls the IL-6/STAT-3/miR-21 signalling pathway, had no effect on FoxP3 mRNA expression. We hypothesize that there are other unidentified factors that affect miR-21 signalling.

IVIG is becoming increasingly common as a treatment for autoimmune and systemic inflammatory diseases, based on its immunomodulatory and anti-inflammatory potential [29]. IVIG can protect against some autoimmune diseases by increasing the number of peripheral T_{reg} cells, but the mechanism of IVIG treatment is unknown [30]. In our study, the decreased expression of miR-155 and over-expression of miR-31 were reversed after IVIG treatment, and the number of T_{reg} were restored. Although our research does not address the mechanism by which IVIG induces the expression of miRNAs, it may provide new avenues to explore the IVIG's immunoregulatory mechanism. Although the importance of miRNAs requires more study, it is also important to note that many important factors are required in the fate determination of cells. In our study, miRNAs increased after IVIG treatment, but not to the levels observed in controls. In contrast, the levels of T_{reg} returned to normal after treatment. Thus, it is likely that IVIG targets other pathways in addition to the miRNAs discussed in this study.

In conclusion, T_{reg} cells involved in the development of KD play a critical role in KD pathogenesis. Our data demonstrate that the down-regulation of T_{reg} may be associated with aberrant miR-155/SOCS-1 signalling and the over-expression of miR-31 in patients with acute KD. IVIG treatment may rescue T_{reg} number and function by regulating miR-155 and miR-31 expression. Due to a limited number of subjects and methods, we were unable to elucidate the specific mechanisms of IVIG treatment in regulating miRNAs in T_{reg} cells. However, our research has provided novel insights into the mechanism of IVIG in the treatment of acute KD.

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None.

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